# The genome-wide screening of yeast deletion mutants to identify the genes required for tolerance to ethanol and other alcohols

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## Abstract

A set of homozygous diploid deletion mutants of the yeast *Saccharomyces cerevisiae* was screened for the genes required for tolerance to aliphatic alcohols. The screen identified 137, 122 and 48 deletion mutants sensitive to ethanol, 1-propanol and 1-pentanol, respectively. A number of the genes required for ethanol tolerance were those also required for tolerance to other alcohols. Numerous mutants with defective genes encoding for vacuolar $H^+$-ATPase (V-ATPase) were cosensitive to these alcohols. A global screening approach of yeast deletion library mutants was useful in elucidating the mechanisms of alcohol tolerance based on different lipophilicities.



## Introduction

Alcohols are generally toxic to microorganisms, as they inhibit cell growth and metabolism. The toxicity of alcohols is strongly correlated with their lipophilicity. The $\log P_{ow}$, defined as the logarithm of the octanol and water partition coefficient of a solvent, has been used as a suitable parameter to estimate the toxicity of alcohols (Salter & Kell, 1995). Lipophilic alcohols with a $\log P_{ow}$ value $> 4$–5 are more toxic to microorganisms than those with lower $\log P_{ow}$ values (Weber & de Bont, 1996). It has been reported that this correlation is applicable to a variety of bacterial strains such as *Escherichia coli*, *Pseudomonas putida* and *Acinetobacter calcoaceticus* (Sikkema *et al.*, 1995; Ramos *et al.*, 2002; Kabelitz *et al.*, 2003). In order to better understand the cellular mechanisms associated with alcohol tolerance, it would be useful to examine the correlation between lipophilicity and the inhibition of cell growth.

Our previous research has also provided evidence that this correlation is applicable to the yeast *Saccharomyces cerevisiae* (Fujita *et al.*, 2004). It was shown that lipophilic alcohols with high $\log P_{ow}$ values were more toxic to yeast than those with low $\log P_{ow}$ values. Furthermore, we have performed morphological and comprehensive gene expression analyses in order to elucidate this correlation. This study revealed that ethanol, 1-pentanol and 1-octanol penetrated the intracellular space and damaged various organelles in a similar way. According to a cDNA microarray analysis, exposure to these alcohols led to a number of up-regulated genes being classified into certain functional categories, and to the grouping of some gene expression profiles into a congeneric hierarchical cluster. These results suggest that yeast cells may deploy a similar defense mechanism against various alcohols with different $\log P_{ow}$ values. In other words, alcohols may exert a cytotoxic effect on analogous targets, regardless of their lipophilicity. In order to elucidate the cellular events of this mechanism, further studies on the genetic pathways and/or mechanisms are needed.

Several genes and pathways required for ethanol tolerance have been carefully studied. Some researchers have suggested that ethanol resistance originates from the lipid composition and fluidity of the plasma membrane (Alexandre *et al.*, 1998; You *et al.*, 2003). Five of the genes, *BEM2*, *PAT1*, *ROM2*, *VPS34* and *ADA2*, identified as required for ethanol tolerance, are related to the integrity of the cell wall (Takahashi *et al.*, 2001). Mutants lacking mitochondrial manganese-superoxide dismutase (MnSOD) are sensitive to ethanol, indicating that *SOD2* is essential for ethanol

tolerance (Costa *et al.*, 1997). However, there is little information regarding the genes required for tolerance to other alcohols. To establish this, a highly efficient and comprehensive analysis is needed.

A quantitative method for analyzing deletion mutants has been developed by the *Saccharomyces* Genome Deletion Project (http://sequence-www.stanford.edu/group/yeast-deletion_project/deletions3.html) in order to assign biological functions to newly identified open reading frames (ORFs). This method is based on the availability of deletion libraries consisting of sets of strains, each of which harbors single deletion alleles covering all known yeast ORFs (Shoemaker *et al.*, 1996). A number of informative screens have been described that are designed to seek deletion mutants sensitive or resistant to a variety of stress conditions, including exposure to oxidants, organic acids and antibiotics (Higgins *et al.*, 2002; Blackburn & Avery, 2003; Mollapour *et al.*, 2004). These screens could be highly efficient tools in determining such genes, or in defining the signaling pathways involved in the tolerance to various alcohols.

In this study, we performed an efficient robotic screen of a set of yeast deletion strains to identify genes required for tolerance to three representative aliphatic alcohols, ethanol ($\log P_{ow}$, −0.30), 1-propanol ($\log P_{ow}$, 0.25) and 1-pentanol ($\log P_{ow}$, 1.51). The results are discussed with respect to the mechanisms of alcohol tolerance, and to the correlation between lipophilicity and the inhibition of cell growth.

## Materials and methods

### Strain and growth conditions

A set of approximately 4500 homozygous diploid strains of *Saccharomyces cerevisiae* which harbored deletions in non-essential genes (the library consisted of 49 plates numbering #301 to #349) constructed in a BY4743 background (*MAT a/MAT alpha his3-1/his3-1 leu2-0/leu2-0 met15-0/MET15 LYS2/lys2-0 ura3-0/ura3-0*) was purchased from Invitrogen Corp. (Carlsbad, CA). Strains were inoculated from stock cultures in 96-well master plates at −80 °C and were grown at 30 °C in YPD medium [1% Difco yeast extract, 2% peptone, 2% glucose (all % w/v)] containing 200 μg mL$^{-1}$ of Geneticin (G418; Sigma, St Louis, MO) and then stored at 4 °C. YPD agar media containing the different alcohols were prepared a day prior to use.

### Screening for alcohol-sensitive deletion mutants

To determine the appropriate concentrations of aliphatic alcohols for screening, 5 μL of serial dilutions of the parent-type BY4743 strain were spotted onto alcohol-supplemented YPD agar plates and incubated at 30 °C for 3 days. Concentrations that did not inhibit growth on the alcohol-supplemented plates were used to screen for alcohol-sensitive deletion mutants, as described below.

Five microlitres of the deletion mutant cell cultures in the 96-well plates were spotted onto YPD agar media supplemented with or without alcohols in multi-trays (128 mm × 85 mm; Asahi Techno Glass Corporation, Tokyo, Japan) using a Biomek® 2000 Laboratory Automation Workstation (Beckman coulter Inc., FL). These plates were then incubated at 30 °C for 3 days. Deletion strains, the growth of which on alcohol-supplemented plates was inhibited relative to their growth on the alcohol-free plates, were scored as alcohol-sensitive. These screens were performed at least twice. Strains exhibiting no growth or growth that was difficult to score were rescreened with serial spotting tests. These tests consisted of preculturing in 96-well plates under the same conditions, followed by spotting 5 μL of serial 10-fold diluted aliquots onto the YPD agar plates, alcohol-supplemented or not. Strains exhibiting no growth were defined as alcohol-sensitive deletion mutants.

All aliphatic alcohols used in this study were purchased from Wako Pure Chemicals Ltd., Osaka, Japan.

### Genome-wide functional analysis

Genes were assigned to functional categories using The Munich Information Center for Protein Sequences, MIPS (http://mips.gsf.de/) and the *Saccharomyces* Genome Database, SGD (http://www.yeastgenome.org/).

## Results

### Determination of appropriate concentrations of aliphatic alcohols for screening

Initially, concentrations of the different aliphatic alcohols to which the parent-type BY4743 strain exhibited sensitivity were determined by a serial dilution spot-test using a range of concentrations up to 15% ethanol, 7% 1-propanol and 1.25% 1-pentanol. BY4743 was observed to not grow on media containing 12.5% ethanol, 5% 1-propanol or 0.75% 1-pentanol (Fig. 1a). Thus, plates containing 10% ethanol, 4% 1-propanol and 0.5% 1-pentanol were used to screen for alcohol-sensitive deletion mutants, as described below.

### Genes involved in tolerance to ethanol, 1-propanol and 1-pentanol

To identify alcohol-sensitive mutants, we screened a collection of approximately 4500 homozygous diploid deletion strains (Fig. 1b). The screen yielded 137, 122 and 48 mutants that were clearly more sensitive to ethanol, 1-propanol and 1-pentanol, respectively, than the parent-type BY4743 strain (Fig. 2). We defined the genes deleted in these mutants as essential for tolerance to these alcohols (Tables 1–3). The list

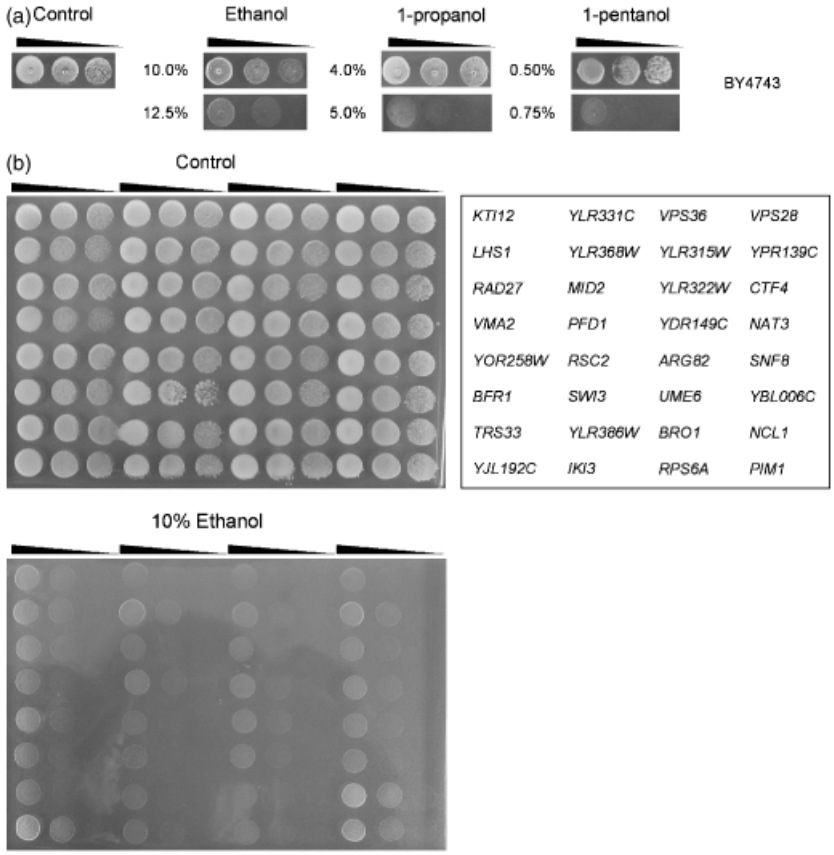


**Fig. 1.** Screening for alcohol-sensitive deletion mutants. The definition of alcohol concentrations for mutant screening (a). The parental strain BY4743 was spotted onto 1% Difco yeast extract, 2% peptone, 2% glucose (YPD) agar media containing different concentrations of ethanol, 1-propanol or 1-pentanol, and grown for 3 days at 30 °C. Shown are representative examples of mutants that conferred sensitivity to 10% (v/v) ethanol (b). Five microlitres of serial 10-fold diluted aliquots were spotted onto YPD agar plates, ethanol-supplemented or not. In this example, all of the strains exhibiting no growth were classified as ethanol-sensitive deletion mutants, except *YJL192C*, *YPR139C*, *NCL1* and *PIM1* deletion mutants.

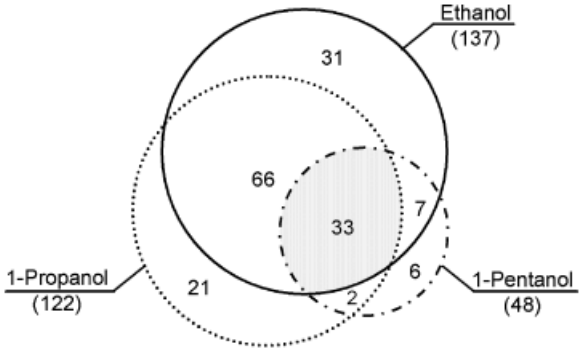


**Fig. 2.** Diagrammatic representation of the distribution of deletion mutants sensitive to ethanol, 1-propanol, and 1-pentanol.

of these genes is available on the website http://kasumi.nibh.jp/∼iwahashi/. On the contrary, no cell growth on media containing 12.5% ethanol, 5% 1-propanol or 0.75% 1-pentanol was observed in the deletion strains (data not shown). This suggests that no deletion enhanced the alcohol tolerance.

We assigned the genes required for alcohol tolerance to a functional category using MIPS and the SGD databases (Fig. 3). The 31 specific genes required for tolerance to ethanol were predominantly categorized based on the cell cycle and DNA processing, protein fate, cellular transport mechanisms, and transcription (Table 1). Some of the genes encoding the protein that forms the protein complex were identified. For example, the cytoplasmic Gim proteins and the heterohexameric cochaperone prefoldin complex were encoded by *GIM* genes such as *YKE2*, *PAC10*, *GIM3*, *GIM4* and *GIM5* (Geissler *et al.*, 1998). We determined that *PAC10*, *GIM4* and *GIM5* were required for tolerance to ethanol. However, *GIM4* and *GIM5* were required for tolerance to 1-propanol and 1-pentanol, respectively. Besides, it is known that Ard1p is a subunit of an N-terminal acetyl transferase; it acts in a complex with Nat1p to catalyze the cotranslational N-terminal acetylation of many yeast proteins (Park & Szostak, 1992). N-terminal acetyl transferases influence multiple processes such as the cell cycle, heat-shock resistance, mating, sporulation, and telomeric silencing

**Table 1.** Classification into functional categories of genes whose deletion confer sensitivity to ethanol

| | |
|---|---|
| Cell cycle and DNA processing (20) | *ANC1, ARD1, BFR1, BIK1, BNI1, BUB1, CNM67, CTF4, ELM1, GRR1, HEX3, HPR1, HTL1, POL32, RAD27, RSC2, SHP1, SHS1, UME6, VID21* |
| Protein fate (20) | *ALG6, DOA4, GIM4, GIM5, LHS1, MFT1, NAT1, NAT3, PAC10, PFD1, PPM1, PRE9, RAD6, TOM37, UMP1, VPS36, VPS41, YME1, YND1, YTA7* |
| Cellular transport mechanisms (17) | *AKR1, APN1, ATP15, BRO1, CLC1, FEN2, FPS1, GTR1, ISA2, LUV1, SHE4, SNF7, SNF8, STP22, TRS33, VPS20, VPS28* |
| Transcription (16) | *CAF16, CST6, CTK3, DHH1, ELP2, ELP6, IKI3, KCS1, PAF1, PAT1, RPB9, SNT309, SRB2, SWI3, TSR2, YAP3* |
| Biogenesis of cellular components (15) | *BEM1, BEM4, BUD27, CWH36, FZO1, HOC1, MID2, NUP120, NUP133, RMD7, SAC6, SMI1, SSD1, TPM1, YIL090W* |
| Vacuolar function (14) | *MEH1, TRP1, VAC14, VMA1, VMA2, VMA3, VMA4, VMA6, VMA8, VMA10, VMA12, VMA13, VMA16, VMA21* |
| Metabolism (6) | *CDS1, CSG2, ERG28, IDP1, TCO89, TRP4* |
| Signal transduction (5) | *ARG82, BCK1, FAB1, RAS2, SLT2* |
| Protein synthesis (3) | *ASC1, RPL13B, RPS6A* |
| Cell rescue, defense, and virulence (3) | *KTI12, SLG1, SOD2* |
| Unknown function (18) | *YBL006C, YDR008C, YDR149C, YDR433W, YEL044W, YGR196C, YHR167W, YKL037W, YKL118W, YLR315W, YLR322W, YLR331C, YLR368W, YML095C-A, YMR003W, YNL080C, YNL133C, YOR258W* |

**Table 2.** Classification into functional categories of genes whose deletion confer sensitivity to 1-propanol

| | |
|---|---|
| Cell cycle and DNA processing (18) | *ANC1, ARD1, BFR1, BIK1, BNI1, CLN3, CNM67, CTF4, ELM1, GRR1, HTL1, JNM1, POL32, SHP1, SHS1, TRF5, UME6, WHI3* |
| Protein fate (15) | *ALG6, BUL1, DOA4, GIM4, LHS1, MFT1, NAT3, PFD1, PIM1, PMT2, PRE9, RAD6, TOM37, UMP1, YND1* |
| Cellular transport mechanisms (17) | *AKR1, APN1, BRO1, FEN2, FIG4, FPS1, GUP1, LUV1, SAC1, SHE4, SNF7, SNF8, SOP4, STP22, VPS20, VPS28, VPS66* |
| Transcription (11) | *ELP2, ELP6, IKI3, NCL1, PAF1, PAT1, RPB9, SNT309, SWI3, TSR2, YAP3* |
| Biogenesis of cellular components (11) | *CWH36, FZO1, GAS1, HOC1, MID2, RMD7, SAC6, SMI1, SSD1, TPM1, WSC2* |
| Vacuolar function (13) | *MEH1, TRP1, VAC14, VMA1, VMA2, VMA3, VMA4, VMA6, VMA8, VMA12, VMA13, VMA16, VMA21* |
| Metabolism (6) | *CDS1, ERG6, GND1, IDP1, PRO1, TRP4* |
| Signal transduction (5) | *ARG82, BCK1, FAB1, RAS2, SLT2* |
| Protein synthesis (4) | *ASC1, RPL13B, RPL20A, RPS6A* |
| Cell rescue, defense, and virulence (4) | *KTI12, SLG1, SOD2, TRM9* |
| Unknown function (18) | *YBL006C, YBL031W, YDR008C, YDR149C, YEL044W, YGR196C, YHR167W, YKL037W, YKL118W, YLR315W, YLR331C, YML095C-A, YMR003W, YNL080C, YNL133C, YOR258W, YPL101W, YPL102C* |

(Polevoda *et al.*, 1999). In this study, *ARD1* was required for tolerance to ethanol, 1-propanol and 1-pentanol. However, *NAT1* was exclusively required for tolerance to ethanol. These results suggest that genes involved in ethanol tolerance are more relevant to the genes encoding the protein that forms the protein complex than to 1-pentanol or 1-pentanol tolerance genes.

The specific genes required for tolerance to 1-propanol ($n = 21$) or 1-pentanol ($n = 6$) were assigned to functional categories such as the cell cycle and DNA processing, cellular transport mechanisms, etc. (Tables 2 and 3). However, the roles of the genes regarding these tolerances were poorly characterized. On the other hand, a large number of deletion mutants that conferred sensitivity to 1-propanol or 1-pentanol were also sensitive to ethanol. Indeed, most of the 1-propanol-sensitive mutants with deleted genes involved in protein fate, cellular transport mechanisms, transcription or unknown functions were also sensitive to ethanol. Furthermore, almost all of the 1-pentanol-sensitive mutants with disrupted genes involved in cell cycle DNA processing were sensitive to ethanol (Fig. 3). Interestingly, several mutants in this group were determined to also be sensitive to other conditions, such as nystatin treatment (*BRO1*Δ, *SNF1*Δ, *STP22*Δ, and *VPS28*Δ) and alkaline conditions (*BCK1*Δ and *SLT2*Δ) (Giaever *et al.*, 2002).

## Genes with cosensitivities to ethanol, 1-propanol, and 1-pentanol

Of the 137, 122 and 48 deletion mutants sensitive to ethanol, 1-propanol, and 1-pentanol, 33 of the mutants also conferred cosensitivity to these alcohols (as shown in the shaded

**Table 3.** Classification into functional categories of genes whose deletion confer sensitivity to 1-pentanol

| | |
|---|---|
| Cell cycle and DNA processing (9) | *ANC1, ARD1, CTF4, ELM1, GRR1, HPR1, HTL1, SHP1, UME6* |
| Protein fate (2) | *GIM5, UMP1* |
| Cellular transport mechanisms (2) | *AKR1, SHE4* |
| Transcription (3) | *ELP2, STB5, TSR2* |
| Biogenesis of cellular components (5) | *BEM1, BUD27, NUP133, RMD7, SAC6* |
| Vacuolar function (14) | *MEH1, TRP1, VAC14, VMA1, VMA2, VMA3, VMA4, VMA6, VMA8, VMA10, VMA12, VMA13, VMA16, VMA21* |
| Metabolism (6) | *CSG2, ERG6, GND1, RPE1, TKL1, TRP4* |
| Unknown function (7) | *YDL172C, YDL173W, YDR008C, YJL120W, YKL118W, YLR315W, YMR003W* |

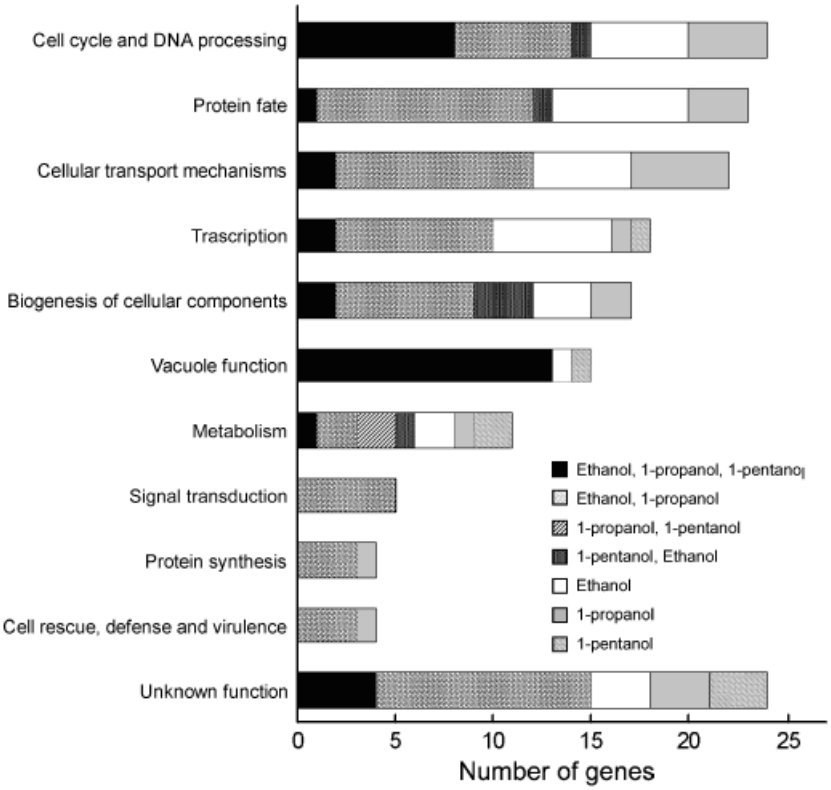


**Fig. 3.** Genetic analysis of the deletion mutants sensitive to ethanol, 1-propanol and 1-pentanol. The *y*-axis shows the biological process categories provided by the Munich Information Center for Protein Sequences (MIPS) and *Saccharomyces* Genome Database (SGD).

part of Fig. 2). These genes were predominately classified into the cell cycle and DNA processing, and the vacuolar function (Fig. 3). Of the eight genes involved in the cell cycle and DNA processing, several genes with which these genes interact were observed to also be required for the tolerance to alcohols (Tables 1–3). Moriya & Isono (1999) reported that *ELM1*, encoding serine/threonine protein kinase, genetically interacted with *DHH1* and *SSD1*. In our results, *ELM1* was required for tolerance to three kinds of alcohol, while *DHH1* and *SSD1* were merely required for the tolerance to ethanol or 1-pentanol.

It should be noted that 13 mutants with deleted genes classified into the vacuolar function category conferred cosensitivity to ethanol, 1-propanol and 1-pentanol (Tables 1–3 and Fig. 3). It is known that the ATP-dependent proton pump called vacuolar $H^+$-ATPase (V-ATPase) acidifies intracellular vacuolar compartments (Stevens & Forgac, 1997). Interestingly, of the 13 deletion mutants, seven genes, *VMA1*, *VMA2*, *VMA4*, *VMA6*, *VMA8*, *VMA13* and *VMA16*, were involved in hydrogen-transporting ATPase activity, the rotational mechanism or its variants (Gene Ontology: 0046961, $n = 7$), and 10 genes, *MEH1*, *VMA1*, *VMA2*, *VMA3*, *VMA4*, *VMA6*, *VMA8*, *VMA12*, *VMA13* and *VMA16*, were involved in vacuole acidification (Gene Ontology: 0007035, $n = 22$). This suggests that the V-ATPase function is fundamentally required for alcohol tolerance.

## Discussion

In order to elucidate cellular mechanisms of adaptation upon exposure to various organic solvents, the $\log P_{ow}$ value has been proposed as a useful indicator of potential toxicity (Sikkema *et al.*, 1995). In this study, we therefore adopted the $\log P_{ow}$ values as the coefficient for lipophilicity of an alcohol, and examined ethanol ($\log P_{ow}$, $-0.30$), 1-propanol ($\log P_{ow}$, 0.25) and 1-pentanol ($\log P_{ow}$, 1.51). It was observed that BY4743 cells did not grow on YPD agar medium containing 12.5% ethanol, 5.0% 1-propanol or 0.75% 1-pentanol (Fig. 1a). These findings indicate that alcohols with high $\log P_{ow}$ values are more toxic to yeast cells than those with low $\log P_{ow}$ values. It was confirmed that the lipophilicity of an alcohol is crucial for cell toxicity.

We observed that 137, 122 and 48 deletion mutants were sensitive to ethanol, 1-propanol and 1-pentanol, respectively (Fig. 2). It is interesting to note that more genes are needed for tolerance to alcohols with lower toxicity, such as ethanol. The number of genes required for tolerance to alcohols seems to be closely related to the lipophilicity (or toxicity) of a given alcohol. Some bypass pathways may work to circumvent the growth-inhibitory effect of alcohols with higher toxicities, such as 1-pentanol. Furthermore, it was observed that most of the deletion mutants that conferred sensitivity to 1-propanol or 1-pentanol were also ethanol-sensitive, and 33 deletion mutants exhibited cosensitivity to ethanol, 1-propanol and 1-pentanol (Figs 2 and 3). These results imply that a certain core set of genes is fundamentally required for tolerance to various alcohols, regardless of their lipophilicity (or toxicity). These issues will be addressed in future studies.

In the present study, mutants with deletions in genes involved in vacuolar function were clearly more sensitive to alcohols than the parent strain, BY4743 (Fig. 3). It is noteworthy that a large number of the 33 mutants that conferred cosensitivity to three different alcohols were lacking genes involved in V-ATPase function (Tables 1–3). Our findings provide evidence that genes involved in the V-ATPase function are required for alcohol tolerance. It has been reported that defects in the V-ATPase function result in the disruption of other cellular processes, including receptor-mediated endocytosis, the maintenance of a neutral pH, the metabolism of nonfermentable carbon sources and the uptake of small molecules (Ho *et al.*, 1993a, b; Munn & Riezman, 1994). The multisubunit complex, yeast V-ATPase, which is composed of a peripheral membrane sector (V1) responsible for ATP hydrolysis and an integral membrane sector (V0) required for proton translocation, is a well-characterized member of the ubiquitous family of electrogenic pumps (Stevens & Forgac, 1997; Inoue *et al.*, 2003). The ATP-dependent proton pumps are coupling the hydrolysis of ATP to proton movement across membranes. This movement results in the acidification of intracellular compartments, such as vacuoles/lysosomes, endosomes and clathrin-coated vesicles (Malkus *et al.*, 2004; Shao & Forgac, 2004). Whereas V-ATPase plays a key role in the maintenance of intracellular pH homeostasis, ethanol is known to reduce intracellular pH values (Rosa & Sa-Correia, 1996). It has been reported that a number of genes involved in ion homeostasis respond to ethanol (Alexandre *et al.*, 2001). For example, *BTN2*, with a putative role in mediating pH homeostasis between the vacuole and plasma membrane $H^+$-ATPase, was up-regulated in response to ethanol (Fujita *et al.*, 2004; Kim *et al.*, 2005). We therefore propose that the genes involved in intracellular pH homeostasis are crucial for tolerance to various alcohols. Contrary to this, some researchers have suggested that intracellular acidification does not account for the inhibition of yeast growth in the presence of ethanol (Rosa & Sa-Correia, 1996). Further analysis is needed to confirm this hypothesis.

Various alcohol-inducible proteins or genes have been identified. For example, it has been reported that sublethal ethanol exposure induces heat shock proteins such as Hsp104 and Hsp30 (Piper, 1995). A global gene expression analysis also had indicated that a large number of genes involved in ionic homeostasis, heat protection, trehalose synthesis and antioxidant defense were up-regulated (Alexandre *et al.*, 2001). In addition, our previous data indicated that numerous genes, up-regulated by ethanol and 1-pentanol, were part of the cell rescue, defense and virulence, as well as the energy and metabolism categories (Fujita *et al.*, 2004). By applying the microarray data to the genes involved in tolerance to ethanol or 1-pentanol, most of the genes were considered to be down-regulated or invariable. Only four of the ethanol-tolerance genes (*ELM1*, *HTL1*, *DOA4* and *YKL037W*) and three of the 1-pentanol-tolerance genes (*TRP4*, *VMA13*, and *YKL118*) proved to be up-regulated (> twofold above control) by ethanol and 1-pentanol, respectively. We also demonstrated that the expression of genes involved in V-ATPase function was mostly down-regulated or invariable in response to alcohols. Conversely, the alcohol-inducible genes mentioned above, such as *BTN2*, *HSP104* and *HSP30*, were not required for alcohol tolerance. It is likely that yeast cells mediate alcohol tolerance by a temporary cell rescue system such as heat shock protein synthesis and/or by ionic homeostasis such as via V-ATPase activity.

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